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Assessing Cold Hardiness in Conifers: A Problem Analysis and Discussion Paper

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1 INTRODUCTION

This report presents a problem analysis of the major cold hardiness tests, methods of evaluating cold injury, and means of expressing cold hardiness in forest seedlings. The ideal testing program should include quantitative measures of cold injury to serve as a logical adjunct to visual damage assessments (Steponkus and Lanphear 1967). The potential methods for measuring cold hardiness in forest seedlings are assessed according to the above criteria to determine the practicality of each testing program.

2 TESTING

The testing process is made up of a series of sequential steps, including:

- selection of sample;
- transportation/storage of test material;
- tissue preconditioning; and
- freeze testing.

2.1 Selection of Sample

Selecting material for frost hardiness testing involves a clear understanding of the question the test should address. Sample selection must consider both the location of the material and the intended focus of the injury evaluations.

2.1.1 Location

Material tested for frost hardiness generally comes from one of two sources:

- field-planted stock (Navratil *et al.* 1986); and
- seedlings from nursery/greenhouse culture (Colombo *et al.* 1984).

The location of the plants scheduled for evaluation often determines the type of test used to measure frost hardiness.

In terms of planted stock, material is usually collected in the field and transferred to the laboratory for testing. Because it is not possible in most cases to transfer whole plants to the laboratory, plant parts, or specific tissues, are collected (Simpson 1983). Dormant shoot segments of current year growth are the most common type of material collected (Sakai 1970; Christersson 1978; Holbo *et al.* 1981; Sakai 1983; Cannell *et al.* 1985). However, detached leaves (Gary 1971; Kedrowski 1979; Biryukova and Kharlamova 1982; Bunce 1985; Abbott *et al.* 1987), buds (Biryukova and Kharlamova 1982), roots (D. Simpson, pers. comm., Jan. 3, 1989), and needle fascicles (Johnson and Gagnon 1988) have also been used as test material.

Another approach used with field-planted material is to propagate the plant by means such as cuttings, and to test the propagule in the seedling, cutting, or graft form (Rehfeldt 1980).

Nursery- or greenhouse-grown seedlings are well suited to the experimental design of frost hardiness testing (Simpson 1983). Because seedlings are grown under controlled conditions, a large, environmentally uniform population is available for sampling, development can be observed on a regular basis, and, therefore, the problems associated with extraneous environmental variables and time-consuming observation and sampling schedules are reduced (Simpson 1983). Although using nursery-grown seedlings for frost hardiness testing is advantageous in controlling environment and reducing sampling costs, extrapolation of findings to mature tree hardiness may be difficult (D. Simpson, pers. comm., Jan. 3, 1989).

2.1.2 Focus of Injury evaluations

Deciding whether injury evaluations will focus on whole plant or organ/tissue damage is essential to designing an appropriate testing regime. However, the main thrust of the experiment may be limited by sample location.

The focus of injury evaluation is important to testing because plant tissues harden and deharden differently (D. Simpson, pers. comm., Jan. 3, 1989). Types of tests fall into two major categories: tests of plant tissues/organs, and whole plant tests (Simpson 1983). Deciding between tests on whole plants or on plant tissues or organs depends on the question to be addressed, the funds available for data collection, and often the personal bias of the researcher (Simpson 1983).

Tests focusing on plant tissues or organs provide detailed information about hardiness differences between tissue types, and are suited to larger sample size tests (Simpson 1983). The main disadvantage to using hardiness tests on specific tissues or organs is that these measures may not relate to the potential of the plant to survive in nature (Simpson 1983; D. Simpson, pers. comm., Jan. 3, 1989). In the field, survivability of planted stock depends on the least tolerant tissue (Simpson 1983). Because low temperature tolerance of tissues varies over the growing season, measuring the hardiness of one tissue many not be a reliable means of assessing actual whole plant hardiness (Simpson 1983; D. Simpson, pers. comm., Jan. 3, 1989). Although relating findings from hardiness tests on plant parts to field survivability is problematic, it is often the only testing means available with field sample material. Designing a test regime which includes both tissue or organ hardiness testing and periodic field observation may reduce this logistical problem. Examples of tests used to assess hardiness in tissues or organs include: freezing bars (Hodges *et al.* 1980), programmed cold baths (Harwood 1981), and differential thermal analysis (DTA) (Christersson 1978). These will be reviewed later in this report.

Whole plant testing is a more common approach when an evaluation of the plant's potential to survive in the field is desired (Simpson 1983). This type of testing is designed to simulate frost events in a controlled environment (Colombo *et al.* 1984). It is generally achieved through the use of a programmable freezer. The freezer run simulates an advective frost (Stathers, 1989) event in which low temperature stress results from cold air around the plant (Simpson 1983). Seedlings in pots or insulated styroblocks are placed into a chamber whose temperature is regulated at a controlled rate to expose the seedlings to reproducible cooling/warming schedules which target a specified minimum (Simpson 1983; Colombo *et al.* 1984; Ritchie 1984). Although whole plant testing is commonly used to evaluate field survival of stock, limitations in relating results from seedling test material to mature trees remain (Simpson 1983).

After the location of sample material and the focus of investigation have been decided, designing a sampling regime is largely a statistical problem (Ritchie 1984). There are, however, several important considerations regarding sample size and frost event scheduling. Sample size should be sufficient to account for genetic variation in phenology as well as experimental error (Ritchie 1984). The sample size should not only satisfy the genotypical and phenological variation in the material sampled, but also the statistical concerns of variability and significance of results.

Frost events may be scheduled on an elapsed time basis; for example, elapsed time from removal from cold storage. This type of scheduling may appear attractive in terms of reducing the interference of variables such as environmental conditions or the developmental status of the stock, but it has the potential to overlook critical points in frost hardiness development (D. Draper, pers. comm., Jan. 1989). Scheduling frost events based on phenology of the test stock (i.e., dormancy, bud swell, bud flush) is more demanding, but it allows the investigator to relate frost tolerance to visible developmental changes (D. Draper and C. Hawkins, pers. comm., Jan. 1989).

2.2 Storage of Material Prior to Testing

Prior to testing, tissue samples collected in the field or greenhouse should be maintained in a dark, 0-2°C environment to prevent the occurrence of premature softening (Sakai and Weiser 1973; C. Hawkins, pers. comm., Feb. 1989). Proper storage ensures that test material is unaltered, with respect to hardening and phenology, from its field status prior to sampling (Aronsson and Eliasson 1970; Aronsson 1975).

2.3 Tissue Preconditioning

2.3.1 Preconditioning to achieve tissue dehardening

To test the effect of natural thaw/freeze events on seedling survival, as occur in chinook conditions during the winter, it is necessary to precondition tissues before freezer testing. In natural warming events in the field, shoot thawing is responsible for the majority of seedling damage (L. Herring, pers. comm., March 1989). Root zone temperatures rarely increase measurably because of the insulative capacity of the soil and snow cover.[1] Subjecting test seedlings to preconditioning regimes that do not differentiate between shoot and root zone exposure, limits the extrapolation of injury assessments to actual field events (L. Herring, pers. comm., Feb. 1989).

Location of stock and climatic conditions during the frost hardiness assessment schedule place several demands on the sampling tissue preconditioning processes. In the winter and preceding spring thaw field conditions, stock must be made accessible for preconditioning and testing (L. Herring, pers. comm., Feb. 1989). One method of ensuring access is to plant test seedlings in multiple replicate pots in the field before there is ground freeze and snow cover (L. Herring, pers. comm., Feb. 1989). At time of testing, pots may be extracted from the field and preconditioned in a greenhouse or growth chamber environment or *in situ*.

Greenhouse/growth chamber preconditioning

The obstacle in simulating thaw conditions in the greenhouse or growth chamber environment, is that it is difficult to differentiate between shoot and root zone conditions. One possibility for maintaining root zone temperatures close to 0°C is to place pots in insulated, waxed boxes (L. Herring and T. Letchford, pers. comm., March 1989). Root zone temperature in the pots may be monitored using a data logger, and compared to air temperature around the shoots. One important consideration in this type of preconditioning process is that relative humidity must be maintained close to the 75% optimum to prevent extraneous seedling damage caused by loss through transpiration of water from foliage (C. Hawkins, pers. comm., Feb. 1989).

Preconditioning in situ

An alternative to greenhouse/growth chamber preconditioning involves exposing test seedlings to thawing temperatures in the field. In this approach the thawing temperatures are taken to field-planted stock via a generator-powered heating device contained in a mobile hood apparatus (L. Herring, pers. comm., Feb. 1989).

The hood is placed over the snow-covered test seedlings in the field, and thawing temperatures are applied to the exposed vegetative growth. Within-hood and root zone temperatures are monitored to ensure appropriate differentiation between shoot and root zone temperature (L. Herring, pers. comm., Feb. 1989). Humidity is maintained, as in natural thaw conditions, through evaporative moisture release from melting snow. Following the desired thaw/dehardening period, pots are removed from the field and tested for frost hardiness. Although this method of *in situ* tissue preconditioning to effect various degrees of dehardening is in the testing phase, it may offer several advantages over conventional preconditioning procedures.

2.3.2 Preconditioning to achieve tissue hardening

In some frost hardiness investigations the objective is to determine the maximum hardiness of a species during a season (Sakai 1966; Sakai and Weiser 1973). This type of investigation involves exposing test material to low temperatures to achieve maximum hardiness prior to freeze testing (Sakai 1966; Sakai and Weiser 1973).

Maximum hardiness may be obtained through natural outdoor hardening (Litzow and Pellet 1980) or exposure to progressively lower temperatures in a programmable freezer (Pellet *et al.* 1981). Extrapolation of maximum hardiness values to actual field frost resistance is an important consideration. To verify that maximum seasonal hardiness values are accurate, results should be

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[1] Herring, L.J. 1987. Winter injury of white spruce in the boreal forest of B.C.: a problem analysis and discussion paper. B.C. Min. For. Res. Rep., Dec. 17, 1987. Unpublished.

compared to frost hardiness assessment results recorded throughout the winter in naturally growing stock (Sakai 1966).

2.4 Freeze Testing

2.4.1 Field testing

Freezing tests may be conducted on field-planted stock through direct observation of naturally occurring frost events (Mergen *et al.* 1974; Nienstaedt 1985; Cannell and Smith 1986). Despite the advantages of extrapolating the results of such investigations to actual field injury, it is difficult to standardize and accurately quantify damage assessments because:

- frost events cannot be separated from the effect of site;
- field conditions are uncontrollable and ability to predict natural events is limited; and
- field observation and sampling is expensive (Simpson 1983).

The limitations of field observation may be avoided by the testing of stock in a freeze chamber (Glerum 1985). Although this type of testing is generally done in the laboratory, measurements using mobile freeze chambers *in situ* have also been conducted (Glerum *et al.* 1966).

2.4.2 Laboratory testing

Laboratory testing is designed to simulate natural frost events while reducing the interference of extraneous environmental effects which may obscure field frost hardiness assessments. As already mentioned, material used for testing is made up of either isolated tissues or intact seedlings (Simpson 1983). Indoor testing is generally conducted in freeze chambers, but liquid baths and temperature gradient bars are also used (Simpson 1983).

Freeze chamber testing

The various types of freeze chambers used in hardiness testing have been reviewed by Warrington and Rook (1980). According to Glerum (1985) the best way to conduct freezing tests is to use a unit with a cam-operated temperature controller. In general, freezing chambers simulate advective frost events (Ritchie 1984). Chambers that deliver both advective and radiative frosts have been developed, but they are less common (Cannell and Sheppard 1982; Ritchie 1984).

Although there are sophisticated freezing units which deliver -70°C (Glerum 1985) for operational hardiness testing, a controller-operated unit equipped to deliver -40°C is adequate (Glerum 1985).

Liquid bath testing

Another method of freeze testing plant organs or tissues is to immerse samples in a controlled temperature liquid bath (Simpson 1983). Alcohol and ethylene glycol are commonly used as the cooling agents in this process (Harwood 1981; Simpson 1983; Christersson *et al.* 1987). Although the liquid bath method delivers an even freeze temperature which can be precisely controlled, it is difficult to use with whole plants (D. Simpson, pers. comm., Jan. 1989). Tissue samples in glass jars or test tubes are generally used in liquid bath testing (Simpson 1983).

Temperature gradient bar testing

Temperature gradient bar testing involves placing tissue samples inoculated with ice in depressions or holes along the length of a metal bar (Simpson 1983). A temperature gradient is created by cooling the bar at one end (Simpson 1983). The disadvantages of this freezing system are that the freeze procedure does not simulate natural frost events, and is only suitable for extremely small tissue samples (Warrington and Rook 1980).

2.5 The Freeze Testing Process

Several aspects of the freeze testing process determine the accuracy and applicability of resultant cold stress assessments. The temperature regime may involve exposing test material to a single temperature minimum (i.e., -18°C frost hardiness test) (Simpson 1983) or to a range of temperatures.[2] The rate of freezing, duration of cold temperature exposure, and conditions for rewarming also affect injury observations (Ritchie 1984).

2.5.1 Point vs. step testing

Most operational frost hardiness testing involves subjecting seedlings to a single temperature minimum during a freezer run (van den Driessche 1976; Glerum 1985). This procedure is repeated over a range of temperatures to bracket seedling frost hardiness (Glerum 1985). An alternative method used by Tinus[3] is designed to simulate a stress response over a temperature continuum. In this procedure, a test group of identical genotypical and phenological origin is divided into temperature subsamples.

Each subsample is removed from the freezer following timed exposure (soaks) at progressively decreasing temperatures. The final subsample is removed following exposure to the lowest temperature in the test sequence. The advantages of this step-wise freezer schedule are as follows:

- All seedlings in the sample are tested at the same point in time, thus reducing variability inherent in multiple freezer run response evaluations.
- The cold temperature exposure and sampling is applied on a continuum, therefore highlighting possible sublethal damage which may be masked in a point test assessment.
- Applying a range of temperatures in a single run reduces the space requirements, freezer run time, and assessment period which is necessary to evaluate frost injury in a series of single temperature cold stress tests (C. Hawkins and D. Draper, pers. comm., Feb. 1989).

Despite the advantages of a step-wise approach to frost hardiness testing, the accuracy of a test in predicting frost hardiness depends primarily on the temperature regime used.

2.5.2 The temperature regime

The temperature regime is composed of the starting temperature, pre-freeze temperature priming, the rate of decrease, the duration of temperature exposure, the rate of rewarming, and the post-thaw conditions (Aronsson and Eliasson 1970; Simpson 1983).

The starting temperature

Prior to frost hardiness testing, it is crucial to expose samples to a standard soak temperature. The purpose of the initial temperature soak is to equilibrate samples in preparation for the test run. In general, this temperature is above freezing and may range from 3°C (C. Hawkins, pers. comm., Feb. 1989) to 10°C (Greer and Warrington 1982).

Pre-freeze temperature priming

Supercooling to as low as -40°C occurs in most deciduous forest trees (Salisbury and Ross 1978). In these plants, ice crystals form extracellularly at sub-zero temperatures. Water drawn from xylem cells increases cell solute concentration and prevents intracellular freezing, which is responsible for cell lysis and death (Salisbury and Ross 1978). Boreal conifers do not deep supercool (Salisbury and Ross 1978). To prevent the occurrence of un-natural supercooling in tissue samples during freezer testing, it is necessary to inoculate them with ice (Simpson 1983). Ice nucleation is achieved by placing damp filter paper (D. Simpson, pers. comm., Jan. 1989), small ice crystals

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[2] Tinus, R.W. 1989. Paper presented at the Stock Quality Working Group meeting. Lake Cowichan, B.C. Jan. 1989. Unpublished.

(Simpson 1983) or moistened cheesecloth (Menzies and Holden 1981; Rietveld and Tinus 1987) in sample jars prior to freezer testing.

Rate of temperature decrease

The rate of temperature decrease must be standard and reproducible between tests (Simpson 1983). Generally, rate of decrease ranges between 5 and 20°C • h^{-1} (Simpson 1983). Cannell and Sheppard (1982) observed no difference in resultant frost injury between 2 and 5°C • h^{-1}. A standard rate of 5 or 6°C • h^{-1} temperature decrease is recommended because higher rates may compound observed injury at the minimum temperature in the freezer schedule (Glerum 1985; C. Hawkins, pers. comm., Jan. 1989).

Duration of temperature exposure

In general, longer exposures to test temperatures increase damage (Ritchie 1984). Glerum (1985) recommends a 1-3 h soak. Cannell and Sheppard (1982) concluded there was little difference in frost injury when tests were conducted at 1- to 4-h exposures, though results from 10- to 30-minute exposures indicated less damage. In multiple temperature freezer runs as described by Tinus,[3] time constraints demand reduced soak durations to complete freezer runs in a 24-h period. The duration of exposure at test temperatures must be constant both within and between freezer runs (Ritchie 1984). Resultant damage may then be compared between runs and at various developmental stages to provide information on probable field responses under similar conditions (Simpson 1983; Ritchie 1984). Although both approaches allow relative comparisons among test material, the former is likely better for predicting tolerance to field-level frost events.

Rate of rewarming and post-thaw conditions

The rate of rewarming can be much faster than the rate of freezing (Glerum 1985). A standard rewarming rate to a constant temperature in the range of 5 to 20°C • h^{-1} is acceptable (Simpson 1983). Following extraction from the freezer, it is essential that all samples be exposed to the same post-thaw conditions before they are placed in the assessment environment (i.e., growth chamber or greenhouse) (Simpson 1983). Samples are commonly stored in a cooler (Colombo *et al.* 1984) or in a dark 0 - 2°C room overnight to allow for gradual thawing (C. Hawkins, pers. comm., Feb. 1989). As with storage of material before testing, post-freezer test storage should maintain a standard environment that will not alter the hardening status or development of the test material before the assessment period (Simpson 1983; C. Hawkins, pers. comm., Feb. 1989).

3 EVALUATING INJURY

The next step in assessing frost hardiness is to evaluate injury resulting from cold temperature exposure (Ritchie 1984). As described by Timmis (1976), methods of assessing tree seedlings for frost hardiness fall into five major categories:

- morphological
- physiological
- chemical
- physical
- electrical.

This breakdown is used here to discuss many of the techniques available for assessing frost hardiness and their potential for operational use.

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[3] Tinus, R.W. 1989. Paper presented at the Stock Quality Working Group meeting. Lake Cowichan, B.C. Jan. 1989. Unpublished.

3.1 Morphological Assessment Methods

Ritchie (1984) states that the only way of unequivocally evaluating frost injury resulting from freezer tests is to hold the seedlings in a greenhouse or growth chamber and then to inspect them visually for damage. Although morphological assessments are subjective and difficult to analyze statistically, they remain the primary method of operational frost hardiness evaluation (D. Simpson, pers. comm., Jan. 1989). Morphological assessments of freezer-tested material include visual inspection of needle, cambium, and bud browning, as well as of root viability, after the plants have been grown for a standard duration in a controlled post-freeze environment (e.g., growth chamber or greenhouse) (Glerum 1985). In general, assessments of one or more of the morphological features listed, in addition to quantitative measures (e.g., physiological and chemical), are used to describe frost hardiness.

3.1.1 Foliage Injury

Visual inspection of foliar browning is commonly used in field and laboratory frost hardiness assessments. In the field, inspection may involve describing foliage browning below a perceived snow line following spring thaw (Friedland *et al.* 1984). In the laboratory, foliage injury in the form of mottling, discoloration from green to olive green or brown, or dryness is usually evident 3 days following testing (Glerum 1985). Index values are commonly used to describe foliar damage (Ritchie 1984; Cannell *et al.* 1985; Glerum 1985). Despite the convenience of such values for scoring injury and analyzing results, indices may not be accurate between species or between seedlings of differing phenology (Ritchie 1984).

In natural field conditions, the type of needle browning changes over a season of growth based on the development of the seedling (Redfern and Cannell 1982). Browning may be in the form of shoot dieback, characteristic of freezing damage before cessation of shoot growth, or more characteristic of autumn field frosts in which damage is confined to current year growth and is in the form of mottling in the centre of needles (Redfern and Cannell 1982). Needles clustered around the apical bud may be unaffected, but in extreme frosts all current year growth may be reddish brown (Redfern and Cannell 1982).

An alternative to assigning index values to rate foliar injury is to describe damage according to percentage browning, by subdivisions of the shoot and subdivisions of the needles. Percentage of needles killed may be summarized in classes and assessed on this basis (Cannell and Sheppard 1982; Colombo and Glerum 1984). This method may be more accurate in describing frost hardiness development because of the variation in tissue susceptibility over a growing season (Cannell and Sheppard 1982).

3.1.2 Cambial browning

Damage symptoms in cambial tissue develop 7 - 10 days after freezer testing (Glerum 1985). To assess the cambium visually, stems are scraped to expose this tissue layer along the length of the stem (Glerum 1985). Damaged tissue is yellow to brown, rather than a healthy green (Glerum 1985). The cambium is rated on a scale to describe the area of tissue affected along the stem (Glerum 1985). In nature, damage to the cambium on the terminal leader may not result in seedling mortality. However, damage at ground line, common in early autumn frosts, is lethal (Timmis and Tanaka 1976). Because hardiness develops from top to bottom of the stem in many conifers over the growing season, description of cambial damage based on portion of stem affected is extremely important (Timmis and Tanaka 1976).

3.1.3 Bud Injury

To assess bud injury, buds are sliced longitudinally and the tissues are inspected for browning (Glerum 1985). Dead or injured buds are brown or tan rather than a healthy green (Glerum 1985). Although the occurrence of some bud mortality may not kill a seedling, greater than 50% damage is often lethal (Glerum 1985).

3.1.4 Root viability

Root viability, although not addressed in many assessment regimes, is an important indication of seedling frost hardiness (D. Simpson, pers. comm., Jan. 1989). Roots are significantly less hardy than the aerial parts of seedlings (Glerum 1985), likely because the roots are buffered by the soil against greater changes in temperature (Glerum 1985). Damage to roots may be evaluated by several methods (D. Simpson, pers. comm., Jan. 1989), but visual inspection of tissue damage under the root bark (Johnson and Havis 1977; Smit-Spinks *et al.* 1983) and root growth capacity determination 7 - 10 days following freezer testing are most common.

Morphological assessment following freezer testing is the most direct method of describing seedling frost tolerance (Ritchie 1984). Although such assessments address operational concerns, results do not necessarily relate to field survival under similar conditions. Foliage death, for example, may or may not affect seedling vigour, and bud mortality may not result in seedling death unless a large proportion of buds are killed. Cambial injury in the terminal may be detrimental to growth but it is not usually lethal, unlike damage at ground level. Root damage may not be lethal unless most or all roots are killed. For these reasons, visual assessments of frost damage should be based on a "whole seedling assessment method" (Glerum 1985). Such a method involves inspection of all of the morphological features mentioned to arrive at a complete description of seedling hardiness (Glerum 1985).

Although morphological assessment is the best method of determining overall seedling response to frost injury, it has several disadvantages:

- Visual assessments are lengthy unless they are completed in an optimum growing environment (i.e., growth chamber) (Ritchie 1984).
- Assessments are subjective and difficult to standardize, particularly with multiple stock types or with material of differing phenologies.
- Results are not directly quantifiable, and therefore data analysis is problematic.

Because of the limitations of visual assessments, particularly of time and accuracy of quantification, several other methods of frost hardiness assessment have been developed. These are often used in conjunction with conventional visual inspection, but they have not replaced it operationally (D. Simpson, pers. comm., Jan. 1989).

3.2 Physiological Assessment Methods

Assessment methods that evaluate the structures or products of physiological systems are common in frost hardiness studies. Few of these methods have been applied operationally in the study of the effects of frost injury in conifers. Two evaluated by Timmis (1976) on Douglas-fir (*Pseudotsuga menziesii*) are:

- infrared gas analysis (IRGA) to measure photosynthesis in whole seedlings; and
- leaf segment floatation to measure relative concentrations of O_2 in intercellular spaces.

3.2.1 Infrared gas analysis (IRGA)

Direct measurements of photosynthesis can be made using whole seedlings, which are preconditioned at 20°C and placed in a closed chamber at 20°C linked to an infrared gas analyzer (Timmis 1976). In an experiment using Douglas-fir seedlings, Timmis (1976) found that rates of photosynthesis, expressed as fractions of photosynthetic rates prior to freeze testing, were as effective as electrical methods of evaluating frost damage (i.e., electrical impedance ratios) when seedling were least hardy (during growth prior to budset under warm, short days). The effectiveness of the IRGA method decreased as seedlings became more hardy (during rest/quiescence under cold, short days and night frosts). For this reason, Timmis (1976) concluded that measurements of photosynthesis using the IRGA method were a poor indication of frost hardiness.

To date, the IRGA method of frost hardiness assessment is not widely used, both because of the high cost of IRGA units and because of the problems associated with measurement methods. Further

refinement of the method to accurately measure hardiness development throughout the growing season is needed before it can be used operationally.

3.2.2 Leaf segment floatation

Truelove *et al.* (1974) found that leaf discs, excised from cotyledons of 10-day old pumpkin (*Cucurbita pepo* L. "Alabama Gold") seedlings, floated on phosphate buffer solutions for a relatively long time only if photosynthesis was unimpaired. They concluded that loss of buoyancy in the absence of photosynthesis was a direct result of depletion of oxygen from the intercellular spaces, followed by gradual filling of those spaces with the buffer solution (Truelove *et al.* 1974). Floatation time of leaf discs or segments may be an indirect measure of frost damage to the photosynthetic apparatus (Timmis 1976).

In a study with 3-mm lengths of Douglas-fir needles, the time taken for 50% of the needle fragments to sink was recorded (Timmis 1976). Leaf segment floatation proved to be a poor measure of frost injury, particularly during rest and quiescence, with only a 61% accuracy in discerning dead from live tissues (Timmis 1976). Periods of needle floatation were closely linked with the ability of needles to photosynthesize, but because of the poor correlation between photosynthesis and frost injury, leaf disc floatation was deemed a poor measure of frost hardiness (Timmis 1976).

The principle that frost injury to plant tissues results in damage to the photosynthetic apparatus — and therefore to a decrease in photosynthesis and formation of its associated products — is well documented. Although the principle of photosynthetic breakdown has been proven, a method of relating decrease in photosynthetic activity to frost injury at varying degrees of tissue hardiness has not been developed. Further refinement of the methods mentioned, particularly direct measurement of whole plant photosynthesis by the IRGA method, are needed before they can be used on the operational level.

3.3 Chemical Assessment Methods

Methods used to assess chemical changes as a result of the breakdown of the photosynthetic apparatus or the inactivation of photosynthetic enzymes, are also used to evaluate the effects of frost injury (Timmis 1976). The following examples have been used to evaluate frost damage in various plant species including conifers:

- enzyme inactivation measured by the tetrazolium chloride test (Rosen *et al.* 1983);
- tissue fluorescence (Öquist and Strand 1985);
- ethane production (Johnson and Gagnon 1988); and
- amino acid leakage measured by the ninhydrin method (Rosen *et al.* 1983).

3.3.1 Enzyme inactivation: the tetrazolium chloride test (TTC)

Many enzymes are inactivated by freezing (Salisbury and Ross 1978). In the presence of an oxidizable substrate, active dehydrogenase in living cells reduces colourless triphenyl tetrazolium chloride (TTC) to insoluble red formazan (Salisbury and Ross 1978). The formazan can be extracted in ethanol and measured by a calorimeter (@530 nm) to determine its concentration in solutions and, therefore, the viability of the tissue sample (Steponkus and Lanphear 1967).

Using 100-mg samples of Douglas-fir needle and stem segments vacuum-infiltrated with TTC and tested according to Steponkus and Lanphear (1967), Timmis (1976) found that measurements of dehydrogenase activity were accurate when tissues were least hardy (i.e., as days lengthen and temperature increases during the period of bud swell to shoot elongation). However, the TTC method did not accurately identify live and dead tissues, as determined by multiple discriminant analysis, as tissues become more hardy (rest to quiescence) (Timmis 1976).

The marginal results reported by Timmis (1976) indicate that the TTC test is useful in dehardened tissues, but inaccurate in detecting true dehydrogenase activity in hardened tissues. Despite the problems associated with the TTC test, preliminary findings suggest this method may be useful with other tissues such as roots (D. Simpson, pers. comm., Jan. 1989).

3.3.2 Tissue fluorescence

Strand and Öquist (1988) found that moderate freezing in darkness of frost-hardened seedlings of *Pinus sylvestris* L. decreased both the quantum yield and the light saturated rate of CO_2 assimilation after thawing. The effect of freezing damage on photosynthetic reactions in herbaceous and frost-hardened conifers may differ because of the increase in frost resistance observed in conifers (Strand and Öquist 1988). Measurements of *in vivo* chlorophyll fluorescence kinetics have been used in a number of studies to assess photoinhibitory damage (Abbott *et al.* 1987; Smillie *et al.* 1987).

Fluorescence is measured by exposing foliage to a trifurcated fibreoptic light system which transmits excitation to, and fluorescence from, needles (Strand and Öquist 1988). During frost hardening of *P. sylvestris* seedlings, a decline in the ratio of variable fluorescence yield (F_V) to initial fluorescence yield (F_O) was measured at room temperature and at 77 K (Strand and Öquist 1988). This decline indicated decreased photochemical efficiency of photosystem II, especially for upper needles (Strand and Öquist 1988). Fluorescence offers several possibilities for evaluating photochemical efficiency before and after cold temperature exposure. Timmis (1976) in similar experiments with Douglas-fir found that fluorescence was a difficult method to use on a large scale. The use of fluorescence in operational applications to assess frost hardiness remains to be proven. Relating photochemical efficiency to projected field survivability has yet to be addressed.

3.3.3 Ethane production

Freezing injury is primarily caused by loss of membrane integrity (Johnson and Gagnon 1988). When membranes are disrupted, free-radical scavenging mechanisms are exhausted, leading to free-radical peroxidation of linolenic acid in cell membranes, and subsequent production of ethane (Johnson and Gagnon 1988). Johnson and Gagnon evaluated ethane production as a method for assessing cold temperature injury in *Pinus taeda* L. seedlings, by comparing it to measurements of diffusate electrical conductivity. Needle samples were exposed to a range of temperatures (0 to -12°C), thawed and incubated at room temperature 1 h before assessment. Following incubation, a 1-mL volume of air was injected into sample vials and gas samples were extracted for ethane analysis by gas chromatography. Immediately after ethane measurements, fascicles were assessed using the electrical conductivity technique (Johnson and Gagnon 1988). Ethane production rates were found to be linearly related to freezing temperature. Production rates were also linearly related to percentage of leaf area frozen, and a greater percentage of cells were killed at each successively lower temperature (every 2°C at 0 to -12°C). In addition, ethane production rates showed good correlation with electrical conductivity measurements in linear regression analysis (Johnson and Gagnon 1988).

MacRae *et al.* (1986) had previously failed to observe ethane production before or after chilling taxa of non-coniferous species. Ethane production showed no relationship to relative chilling sensitivity either within related groups or between genera and families (MacRae *et al.* 1986).

Further investigation of the ethane production method of frost hardiness assessment is needed before it can be used operationally. The findings of Johnson and Gagnon (1988) indicate further studies on coniferous species are warranted.

3.3.4 Amino acid leakage: the ninhydrin method

As a result of membrane disruption caused by frost injury, amino acids and other compounds may be released from the cell (Salisbury and Ross 1978). Ninhydrin-reactive compounds react with free amino acids, thereby indicating cell leakage (Salisbury and Ross 1978). The ninhydrin test is based on this principle and has been used to assess damage in various plant tissues, most recently roots (Rosen *et al.* 1983; D. Simpson, pers. comm., Jan. 1989).

3.4 Physical Assessment Methods

Physical assessment methods for determining frost injury as summarized by Timmis (1976) involve the measurement of plant water status. Two aspects of plant water status in frost-damaged tissue, and methods used to record them, are addressed here:

- xylem pressure potential and transpiration measurement using the pressure bomb (Kincaid and Lyons 1981; Grossnickle and Blake 1986; Grossnickle 1988 a, b) and diffusion porometer (Blake 1983; Graham and Running 1984); and
- extracellular ice formation measured using nuclear magnetic resonance (NMR) and differential thermal analysis (DTA).

3.4.1 Xylem pressure potential and transpiration analysis

Measurement of xylem pressure potential to determine cold hardiness in plants is based on the principle of varying tension in xylem water caused by membrane fractionation (Bixby and Brown 1974). When cells are frozen intracellularly, membrane fractionation likely occurs along with a rapid loss of cellular water (Bixby and Brown 1974). As xylem water content decreases, xylem pressure potential becomes more negative (Salisbury and Ross 1978). An increase in cell rupture and death of tissues caused by frost injury should result in an increase in xylem pressure potential (less negative) (Bixby and Brown 1974).

In studies with black locust seedlings, Bixby and Brown (1974) recorded obvious changes in xylem pressure potential between seedlings frozen above and below their degree of cold hardiness. Temperatures at which marked changes in pressure potential were observed correlated with temperatures at which survival was longer term. From their findings, Bixby and Brown concluded that the pressure chamber could be a rapid and useful tool in evaluating seedling hardiness and response to frost injury.

Since Bixby and Brown's study, xylem pressure potential has been used to evaluate performance of newly planted stock (Grossnickle 1988 a and b) and to investigate winter water relations in natural and introduced field stock, which commonly shows susceptibility to winter drought stress (Cowling and Kedrowski 1979; D'Aoust and Hubac 1986).

Timmis (1976) used the pressure chamber technique to measure xylem water potential in detached branches of Douglas-fir after 2 h of light preconditioning. Plant water stress measured by xylem pressure potential proved to be an inaccurate method of discerning live from dead tissues, with an average success of only 71% (Timmis 1976). Along with needle floatation, xylem pressure potential was found to be the least reliable measure of frost damage, particularly in hardened tissues (Timmis 1976). Timmis concluded that plant water stress gave poorer predictions of water stress than observed by Bixby and Brown (1974).

Transpiration analysis, mainly in the form of needle conductance measurements made with a steady-state porometer, often accompanies xylem pressure potential measurements in evaluating plant water stress (Grossnickle and Blake 1985; Grossnickle 1988 a and b). Graham and Running (1984) observed a general decrease in maximum daily leaf conductance from dehardening in the spring to the summer.

The use of the pressure bomb and steady-state porometer in determining hardiness of conifer seedlings has shown mixed results. Although initial investigations indicated a strong correlation between xylem pressure potential and membrane fractionation and cell water loss, more recent studies have indicated problems with this method, particularly in hardened tissues (Timmis 1976). Further investigation into the role of plant water stress measurements to assess frost injury is needed before methods such as these can be used successfully to evaluate frost hardiness over the entire growing season.

3.4.2 Extracellular ice formation

Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) is a direct method of studying freezing in plant tissues (Burke *et al.* 1976). It is a type of spectroscopy which detects freezing in tissues by measuring large spectral differences between ice and liquid water. Ice and solids are not observed on conventional NMR spectrophotometers because they have wide lines (30 000-100 000 Hertz). Liquid water has narrow line widths (one to several hundred Hertz) and shows up clearly. The area under the NMR curve is proportional to the amount of liquid water. As freezing progresses in a sample, the NMR line becomes progressively broader until the peak is of low amplitude.

In theory, NMR is a possible method of detecting frost hardiness in plant tissues. However, several attempts to correlate the quantity of unfreezable or bound water (determined from comparisons of control and frozen tissues) with cold hardiness have been unsuccessful (Burke *et al.* 1976). The cost of obtaining equipment also restricts the use of NMR for frost hardiness assessments.

Differential thermal analysis (DTA)

Calorimetry involves the measurement of exothermic (heat releasing) and endothermic (heat consuming) events by recording relative changes in temperature in a tissue sample and a non-living reference (Burke *et al.* 1976). When water freezes (an exothermic reaction), heat production can be measured to characterize tissue freezing in plants. Water exotherms, or heat-consuming reactions, can be studied similarly during thawing. Although most deciduous species exhibit deep supercooling, during which ice forms extracellularly between cells and therefore cell injury is avoided, this phenomenon is not observed in boreal conifers.

The DTA procedure involves placing two thermocouples, wired in series, into both a killed sample and a live tissue sample (Salisbury and Ross 1978). The differential temperature of the samples is monitored on a chart recorder as they are frozen to -40°C and below in a closed chamber (Burke *et al.* 1976). An initial spike at -5 to -10°C is the high temperature exotherm (HTE) and represents heat of fusion release from the freezing of extracellular water (Salisbury and Ross 1978). When xylem ray cells freeze, the released heat of fusion results in a sharp rise in temperature recorded as a peak. This second peak, occurring between -10 and -45°C in supercooled species, is thought to represent the low temperature exotherm (LTE) in which heat of fusion is released by the freezing of supercooled intracellular water (Salisbury and Ross 1978).

The temperature at which LTE appears is thought to represent a precise, quantitative estimate of cold hardiness (Tinus *et al.* 1985). The DTA procedure has proven to be useful with Douglas-fir and Englemann spruce (Tinus *et al.* 1985), *Picea abies* (Salai *et al.* 1984; Pukacki 1987), and various other genera of *Abies*, *Picea*, *Tsuga*, and *Cedrus* (Sakai 1979). However, despite the favourable results obtained using DTA to evaluate frost hardiness in buds and foliage of a wide variety of conifers, the method can not be used with boreal and high elevation species that do not exhibit deep supercooling. Further development of DTA is needed to provide a reliable means of assessing cold hardiness in plant tissues. Investigation of freezing processes in boreal and high elevation species may prove helpful in establishing a method of exotherm analysis and detection of lethal temperature in this group.

3.5 Electrical Assessment Methods

Various electrical assessment methods have been developed for use in evaluating frost hardiness in plants. They include:

- the oscilloscope square wave method (OSW) (Holbo *et al.* 1981; Racey 1984; Day *et al.* 1985);
- the electrical impedence method (EI) (van den Driessche 1973; Glerum 1985; Rikala and Repo 1987); and
- the electrical conductivity technique (EC) (Colombo *et al.* 1982; Colombo *et al.* 1984; Colombo and Hickie 1987; Zhu and Liu 1987)

In general, electrical assessment methods for determining frost hardiness are used in a greater number of practical applications with nursery stock than are the physiological, chemical, or physical methods previously mentioned. Several of these methods are part of integrated programs used to assess frost hardiness in operational settings.

3.5.1 Oscilloscope square wave (OSW) method

The oscilloscope square wave method of determining hardiness is based on a physiological change in stem tissues as stock hardens in the fall (Day *et al.* 1985).

The technique involves impaling terminal shoots of seedlings with a four-pronged electrode coupled to a function generator which emits an electrical "square wave" to an oscilloscope, which in turn displays the signal (Day *et al.* 1985). The principle of this method is that the pattern of the square wave resulting from transmissions at 1.0 kilohertz (kHz) through the stem is modified according to the hardiness of the stock. The OSW method has been used operationally at the Thunder Bay Forest Station since 1978 on white and black spruce.

According to Day *et al.* (1985), the OSW method has several advantages over other electrical methods of assessment in that it does not measure resistance directly and, therefore, is not subject to the problems associated with variable stem diameter, or effectiveness of electrode contact. In general, as tissues become increasingly hardy towards fall, the wave trace changes from a right-hand trace (which peaks in the middle frequencies), through to a relatively flat trace (transitional wave trace) and then to a left-hand trace (which peaks at low frequencies). Lifting is scheduled according to definite "crossover" from a right- to a left-hand trace.

Despite the correlation cited by Day *et al.* (1985) between the square wave pattern and hardiness status of nursery stock, previous studies showed that ability of the OSW method to measure hardiness was questionable. Racey (1984) noted that because wave patterns are temperature dependent, measurement in the field is not feasible. Temperature correction factors are not possible because of the electrical theory involved and the subjectiveness of interpretation of results.

The interpretation of the wave form is also questionable in that not all species and age classes behave in the same manner while in identical physiological states (Racey 1984). Holbo *et al.* (1981) found that early season readings lacked consistency, which made prediction of lifting time impossible. They concluded that the oscilloscope technique provides little practical insight about hardiness development.

Results of long-term application of this method at installations such as the Thunder Bay Forest Station will likely provide more information on the practical use and predictive ability of the OSW technique.

3.5.2 Electrical impedence method

Electrical impedence has long been identified as a factor which changes with the vigour and physiological state of seedlings (Racey 1984). Electrical impedence is associated with alternating current and is a result of electrical resistance plus capacitive reactance (Timmis 1976). An increase in ionic permeability of frost-injured cell membranes can be measured by a decrease in electrical impedence of stem tissue.

Measurements of electrical impedence at 1 kHz taken before and after freezing have been used to detect tissue damage (Racey 1984). A more rapid approach based on measuring an impedence ratio (IR) of kilocycle over megacycle impedence (kHz/MHz) after freezing has also been developed (Glerum 1970). The IR method is based on the principle that impedence at 1 kHz decreases with increasing injury, while impedence of living and dead tissues are nearly equal when measured at 1 Mhz. The IR is little affected by the type of conifer stem tissue measured, compared with the single frequency impedence (van den Driessche 1973).

The electrical impedence method uses an electrical impedence meter connected by wires to two parallel steel pins which act as electrodes (Ritchie 1984). The electrodes are inserted into the lower stem (just above the root collar) of the seedling and a small voltage is applied (van den Driessche 1973). Cold temperature injury is indicated when measurements of IR decrease from approximately 4 to 1.

Timmis (1976) found that electrical impedence measurements of frost damage were consistently reliable, with between 88 and 92% of the Douglas-fir seedlings correctly classified as alive or dead. In addition, estimates of temperature lethal to 50% of the population (LT_{50}) were made within 1°C at any phase of hardening or dehardening (Timmis 1976).

The accuracy of the electrical impedence method has been reported by many researchers (Glerum 1970; Blazich *et al.* 1974; van den Driessche 1973; Ritchie 1984; Bates 1988). However, interpretation of IR values is difficult (Racey 1984).

Impedence varies with stem diameter non-linearly (Racey 1984). Readings taken higher up the stem have been shown to be more accurate than those closer to the root collar (Timmis 1976). Because impedence also varies linearly with temperature, field measurements can be inaccurate (Racey 1984). Correction factors for diameter and temperature must be calculated and incorporated into final IR values.

Electrical impedence also depends on the physiological condition of seedlings evaluated (Ritchie 1984). During early hardening until late November, LT_{50} values can be estimated within 1°C if an IR of 3 is used to discriminate between live and dead seedlings. After November, the IR increases to approximately 5 to discriminate between live and dead tissues. The IR values are less reliable in mid-winter because low temperatures affect buds before stems, and bud injury is not detected by stem impedence ratios. Bud mortality must be assessed separately to evaluate seedling injury.

Although the electrical impedence method is rapid (results available in 24 h) and non-destructive, problems in interpreting IR values remain. Further investigation of this method, focusing on the interpretation of IR values at different stages of hardening and dehardening, are necessary.

3.5.3 Electrical conductivity method

The electrical conductivity or diffusate conductivity method is based on the principle that frost-injured plant cells exhibit membrane disruption which allows cytoplasmic material to escape into the xylem (Ritchie 1984). The cytoplasm contains dissolved material and has a higher electrical conductivity than apoplastic water, which is relatively pure. By comparing the electrical conductivity of xylem diffusates from uninjured, frost-injured, and killed seedling tissues, the amount of injury that occurred can be estimated.

The electrical conductivity (EC) technique as described by Colombo and Hickie (1987) is as follows. Test seedlings are divided into two groups, one of which becomes a non-frozen control, while the other is exposed to temperature treatments in a programmable freezer. Whole seedlings may be frozen in styroblocks and shoot samples taken following a 24-h thaw period (C. Hawkins, pers. comm., Feb. 1989), or the shoot tips may be excised and frozen in jars (Colombo *et al.* 1984; Colombo and Hickie 1987). Initial EC measurements are made on both control and thawed temperature-treated shoot tips 24 h following immersion in distilled water (Colombo and Hickie 1987). Shoot tips are replaced in the sample jars and killed in a 90°C oven for 4 h. Twenty-four hours following the oven-kill, an EC kill is taken on both the control and the frozen samples. Frost hardiness is determined with the electrical conductivity technique using an Index of Injury (I_t) value that is a measure of relative electrical conductivity differences in control, frozen, and killed tissues (Colombo and Hickie 1987). Detailed descriptions of sampling and measurement are given by van den Driessche 1969; Colombo *et al.* 1984; and Colombo and Hickie 1987.

Use of the EC method is gaining popularity in operational frost hardiness testing (Colombo *et al.* 1984). The EC measurement has been found to indicate accurately frost injury in Monterey pine (Ritchie 1984), black spruce (Colombo *et al.* 1984), jackpine (Colombo and Hickie 1987), and white spruce (Colombo *et al.* 1982). However, other researchers have found the method to be of limited use because, although the lethal temperature of whole plants subjected to freezing temperatures could be predicted well, critical indices of injury could not be identified (Ritchie 1984).

Electrical assessment methods offer several advantages over physiological, chemical, and physical methods of frost hardiness assessments. They are rapid and sensitive and have been shown to predict accurately frost hardiness in operational applications. Although the electrical methods discussed have shown practical use, interpretation of injury values is problematic and depends on species, age, and physiological state. Other variables such as stem diameter (OSW and EI methods) and temperature (OSW, EI, and EC methods) must be corrected so that accurate injury values can be obtained. Further investigation and development of these methods should solve some of the interpretational problems associated with these techniques.

4 EXPRESSING COLD HARDINESS

Once freeze testing has been evaluated, it is necessary to express cold hardiness in a standardized, quantitative form. Because hardiness testing is measured with a diverse group of evaluation methods, arriving at a standardized expression of hardiness between tests is impossible. Within a single assessment regime, however, summarizing results according to acceptable methods is essential. Means of expressing cold hardiness from various evaluation methods include:

- LT_{50} estimates;
- visual injury indices; and
- logistic regression models.

4.1 LT_{50} Estimates

LT_{50} estimates are calculated according to the test temperature shown to result in injury or death of 50% of a test population (van den Driessche 1976; Ritchie 1984; Navratil *et al.* 1986). Although this expression can be used with assessments on specific organs or tissues, whole plant estimates are more common (D. Simpson, pers. comm., Jan. 1989). The LT_{50} values are interpolated from a graph of percent damage versus test temperature (Glerum 1985). Lethal temperatures for any percentage damage may be obtained from the same graph (e.g., LT_{20}, LT_{10}).

4.2 Visual Injury Indices

To standardize and summarize visual injury evaluations, index values can be used to describe varying degrees of injury (Ritchie 1984). Such injury index values have been employed by many investigators (Menzies and Holden 1981; Cannell and Sheppard 1982; Colombo and Glerum 1984; Glerum 1985; Jonsson *et al.* 1986), but most of these are specific to seedlings of a given age, species, and physiological state. Injury indices should be designed with these considerations in mind. Index values of cold temperature damage are most descriptive when they incorporate a range of morphological features as described in Glerum's (1985) review of the "whole seedling assessment method" used by the Industrial Forestry Association, Toledo, Washington. In this method seedlings are assigned a viability rating based on pooled injury indices describing needle, bud, and cambial damage (Glerum 1985).

The disadvantages of injury indices are that they are:

- specific to the given species, age, and physiological state of a test population;
- subjective, and may mask potential sub-lethal damage; and
- difficult to analyze statistically because a single index value often represents a wide range of injury observations.

Because of the subjective nature of visual injury index values, quantifiable expressions of cold hardiness obtained from electrical or chemical assessment methods are often incorporated into evaluation regimes.

4.3 Logistic Regression Models

In some cases, LT_{50} values and similar expressions of injury do not account for all of the interactions within a test population. In a study of cold acclimation in *Pinus contorta*, Rehfeldt (1980) found that analysis of variance for assessing differences in hardiness among populations on multiple sampling dates did not remain constant through cold temperature acclimation. To assess the differences in hardiness of populations, percentage injury data were fitted to a logistic regression model (Rehfeldt 1980). Multiple regression analysis allowed variation in cold hardiness among populations to be related to genetic differentiation, elevation, and geographic region.

5 LABORATORY TESTING: EXTRAPOLATION TO FIELD CONDITIONS

The objective of laboratory frost hardiness testing — to simulate and assess the effects of cold temperature exposure under controlled conditions — facilitates the study of frost injury. At the same time, however, it puts limitations on the ability to model actual field conditions. Extrapolation of laboratory test data to seedlings or mature trees in the field is the major difficulty in assessing frost hardiness (Ritchie 1984).

Possibilities exist for relating laboratory results to actual field performance. Again, frost hardiness studies on identical field-planted stock in conjunction with laboratory testing is recommended. Outplanting studies completed on previously assessed stock are useful in determining whether laboratory testing shows actual predictive value in describing field survivability. Frost hardiness testing schedules based on developmental or physiological parameters (i.e., stock phenology), rather than on an arbitrary time scales (e.g., days following removal from cold storage or days following plant), provide more useful measurements of frost hardiness.

Until further studies relating laboratory frost hardiness testing to subsequent field performance of stock are completed, a reliable method of extrapolating test data to field conditions is not possible. By considering the advantages and disadvantages of the various methods, along with the experimental goals and resource availability, it should be possible to design a testing program which accurately assesses the cold hardiness of nursery stock.

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